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Csk Homologous Kinase, a Potential Regulator of CXCR4-mediated Breast Cancer Cell Metastasis

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14. ABSTRACT

Metastasis is still a major cause of morbidity and mortality in breast cancer patients. However, current therapy still fails to kill migrating (metastasizing) breast cancer cells. CXCR4 is a chemokine receptor implicated in the metastatic migration of breast cancer cells and its expression predicts poor prognosis in breast cancer patients. Several studies have revealed that the block of CXCR4 functions prevented metastasis in breast cancer. However, CXCR4 is expressed on the surface of several tissues and there are some concerns about using drugs to target CXCR4. In fact, AMD-3100, a CXCR4 receptor blocker, exerted unfavorable side effects in a clinical trial. Therefore, it is necessary to develop tissue-specific and tumor-specific CXCR4 blockers. Because Csk Homologous kinase (CHK) is a negative regulator of CXCR4 and preferentially expressed in breast cancer cells, modulating CHK activity may facilitate the development of side-effect-free treatment for metastatic breast cancer. Our aim is to identify the functional role of CHK in breast cancer metastasis using *in vivo* xenograft model systems. With resulting information from this study, we may devise treatments that selectively target metastatic breast cancer cells.

15. SUBJECT TERMS
Breast Cancer Metastasis, CSK homologous kinase, CXCR4

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Introduction

CXCR4 and Metastatic Breast Cancer

Earlier diagnosis and development of new drugs significantly improved survival for breast cancer patients. However, the metastatic migration of breast cancer cells is an immediate concern for breast cancer patients as it remains the actual cause of morbidity and mortality. The expression of chemokine receptors, such as CXCR4 and CCR7, is tightly correlated with the metastatic properties of breast cancer cells (1). This study by Muller et al showed that the level of CXCR4 is higher in malignant breast tumors than in their normal healthy counterparts, suggesting that its expression level correlates with increased metastasis-associated mortality. In vivo, neutralizing the interaction of CXCR4/CXCXL12 significantly impaired the metastasis of breast cancer cells and cell migration (1). Furthermore, Kao et al. (2) have shown that the expression of CXCR4 in surgically resected invasive ductal carcinomas (n=79) is significantly correlated with the degree of lymph node metastasis. Another study has also described that breast cancer cells metastasized to the lungs express very high levels of CXCR4 as compared with the parental cells, suggesting the pivotal role of CXCR4 in breast cancer cell metastasis (3). These results are further substantiated by the fact that CXCR4 is one of the few genes that is upregulated in bone-metastasized breast cancer cells (4). Consistent with these studies, knockdown of endogenous CXCR4 gene expression in breast cancer cells resulted in significant inhibition of breast cancer cell migration in vitro (5).

Taken together, CXCR4/CXCL12 signaling axis is a major driving force behind the metastatic migration of breast cancer cells and might be an attractive target for future therapy for metastatic breast cancer. However, CXCR4 is expressed on the surface of several tissues and there have been some concerns about using drugs to target CXCR4. Non-tissue specific blocking of CXCR4 deficieint mice displayed serious heart and artery problems suggesting that antagonizing the signalling activity of CXCR4 might have severe side effects in vivo (6). In fact, AMD-3100, a CXCR4 receptor blocker, exerted unfavorable side effects in the clinical trial developing gastrointestinal side effects, thrombocytopenia, and atrial and ventricular arrhythmias and the study was stopped (7). Accordingly,

the CXCR4 blockers are not allowed to be used in the clinical practice and the drug is not likely to move forward in its current formulation. Therefore, it is necessary to develop a strategy to interfere CXCR4/CXCL12 signaling axis selectively in target tissues, such as cancer cells.

Csk homologous kinase(CHK) and CXCR4

Activation and dysregulation of signaling pathways often leads to tumorigenesis. Of signaling pathways, protein tyrosine kinase activity is closely associated with tumor progression and malignancy. Abnormalities of tyrosine phosphorylaton can lead to hyper-proliferative disorders, such as cancer and lymphoma. Csk homologous kinase (CHK) is a non-receptor tyrosine kinase and a second member of the Csk family. Like Csk, CHK has Src homology 2 (SH2) and SH3 domains and lacks the consensus tyrosine phosphorylation and myristylation sites found in Src family kinases. CHK has been shown to phosphorylate the C-terminal negative regulatory tyrosine residue of Src family kinases (e.g. Lck, Fyn, c-Src and Lyn) in vitro and in a yeast co-expression system suggesting that CHK may share functional properties with Csk (8). Despite its structural similiarities, CHK displays unique molecular functions. While CHK knockout mice didn't show any apparent abnormal phenotype, Csk KO mice showed a defect in the neural tube formation and died at E11.5. (9).

CHK has been suggested to play a specific role as a novel negative growth regulator of human breast cancer on the basis of the following observations: 1) Unlike Csk, which is ubiquitously expressed, CHK is specifically expressed in primary breast cancer specimens, but not in normal breast tissues (10-12). 2) Unlike Csk, which cannot associate with ErbB-2, CHK binds directly to phospho-Tyr1248 of the ErbB-2/neu receptor kinase upon heregulin stimulation and inhibits Src kinase activity (11). 3) Substantial evidence supports a role for CHK as a negative growth regulator of human breast cancer through inhibition of ErbB-2/neu-mediated Src-family kinase activity. 4) The tumor growth of wild type CHK-transfected breast cancer cells in nude mice is significantly inhibited as compared to that of the nontransfected cells or cells transfected with kinase-dead CHK (12). 5) Overexpression of CHK in breast cancer cells markedly inhibits the cell growth, transformation, and invasion induced by heregulin, and also causes a significant delay of cell entry into mitosis (12). Thus, CHK not only inhibits breast cancer cell proliferation and transformation but also may inhibit tumor cell invasion, suggesting its possible role in cell motility and metastasis in breast cancer.

Our previous results showed that CHK regulates CXCR4 expression (*13*). Because CHK is preferentially expressed in breast cancer cells but not in normal breast cells (70 out of 80 breast carcinoma specimens) (8), it is anticipated that inhibiting or enhancing CHK activity modulate CXCR4 expression selectively in breast cancer cells and may facilitate the development of side-effect free treatment for metastatic breast cancer.

Body

Task 1. To assess the role of CHK kinase activity in metastatic migration of breast cancer cells using xenograft model:

a. ***Assess CXCR4 expression in wild type (wt) CHK- and dead kinase (dk) CHK-transduced breast cancer cells***

Among the breast cancer cell lines that we have tested to date, MCF-7 and MDA-MB361 breast cancer cells showed a good correlation between the expression of CHK and CXCR4 mRNA expression (Figure 1). From these data, we were able to select two cell lines; MDA-MB361 and MCF-7. We determined whether CHK regulates the cell surface expression of the functional CXCR4 receptors in MCF-7 and MDA-MB361 cells. Whereas CHK overexpression induced CXCR4 mRNA, we couldn't observe a significant increase of CXCR4 protein in MCF-7 cells (data not shown). While we found a relatively good correlation between CXCR4 mRNA and protein in MDA-MB361 cells, they grow very slowly upon CHK-transduction, which makes us difficult to obtain enough cells for transplantation. To perform in vivo experiment as proposed in Task 1b and Task 2c, it is necessary to establish another CHK-expressing breast cancer cell line. A newly established cell line, CHK-transduced SKBR-3, grew at a faster rate than CHK-transduced MDA-MB361 cells. We will test whether there is a good correlation between CXCR4 mRNA and protein levels in this cell line.

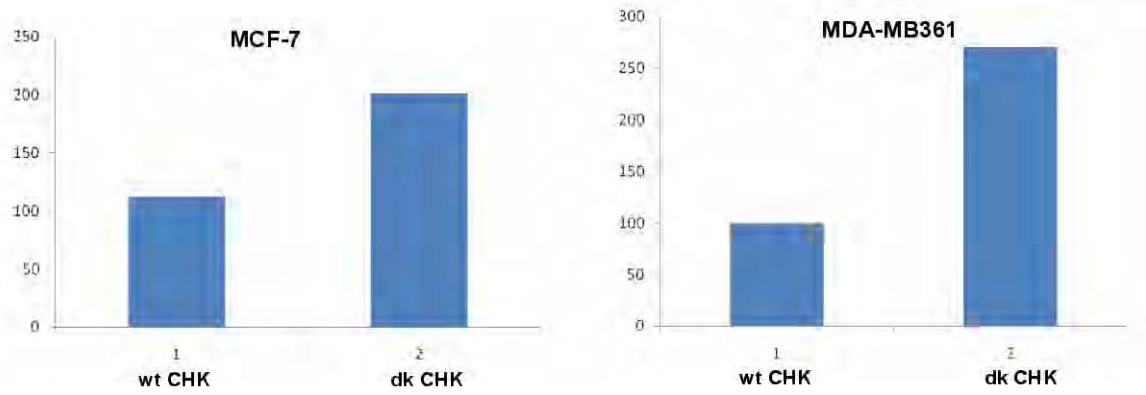

Figure 1. Expression profiles of CXCR4 in CHK expressing breast cancer cell lines

Total RNA was isolated from the indicated cell lines. Aliquots of cDNA were amplified with human CXCR4 and human GAPDH primer sets. The CXCR4 expression was normalized to GAPDH by subtracting their Ct values from the GAPDH Ct value. Values are based on delta-delta-Ct values and represent the median percentages compared to the wild-type CHK value (basal set to 100%). wt CHK: wild-type CHK-transduced cells, dk CHK: dead kinase CHK-transduced cells.

b. ***Transplant wtCHK- and dkCHK-transduced breast cancer cells into female NOD/SCID mice***

Thirty nine NOD/SCID IL-2γ null mice are needed as recipients. NOD/SCID IL-2γ null mice were bred in our own animal facility and we now maintain them in sufficient numbers to perform the xenotransplantation. During the extension period, wild-type and mutant CHK-transduced SKBR-3 cells will be expanded. Once CHKs-transduced cells (from Task 1 a) are expanded into the large number of cells required for transplantation, we will transplant these cells into NOD/SCID IL-2γ null mice by tail vein injection.

c. ***Assess for the presence of metastasis at 7-8 weeks post transplantation and track the transplanted breast cancer cells using imaging system***

It takes approximately 6-8 weeks for engrafted SKBR-3 breast cancer cells to metastasize to other organs. Therefore, recipient mice (from Task 1.b) will be screened by IVIS 200 imaging system at 6-8 weeks post transplantation.

Task 2. To assess the ability of CHK kinase enhancer/inhibitor to modulate YY1 binding to CXCR4 promoter and CXCR4-mediated breast cancer cell migration

a. Test whether CHK SH2 domain binding mutants, G129R and R147A regulate CXCR4 expression

We successfully completed construction of MSCV-retroviral vectors encoding either wild-type CHK or kinase-dead CHK or wild type SH2 domain or SH2-R147A or SH2-G129A. All these constructs were confirmed by DNA sequencing. When breast cancer cells were transduced with these retroviral constructs, high levels of GFP expression were exhibited in cells, indicating high efficiency of transduction (Figure 2a).

Interestingly, wild type SH2 domain-transduced cells expressed a significantly higher level of CXCR4 mRNA compared with cells overexpressing full length CHK. Furthermore, the level of CXCR4 mRNA in G129R-transduced cells was lower than that of R147A-transduced cells (Figure 2b). These results support our working hypothesis that truncated forms of the CHK act as more potent regulators of CXCR4, and their small size may allow for improved penetration into breast cancer cells.

To verify the cell surface expression of CXCR4 in CHK-transduced cells, we will perform flow cytometry analysis on CHKs-transduced breast cancer cell lines (MCF-7, MDA-MB361, and SKBR-3).

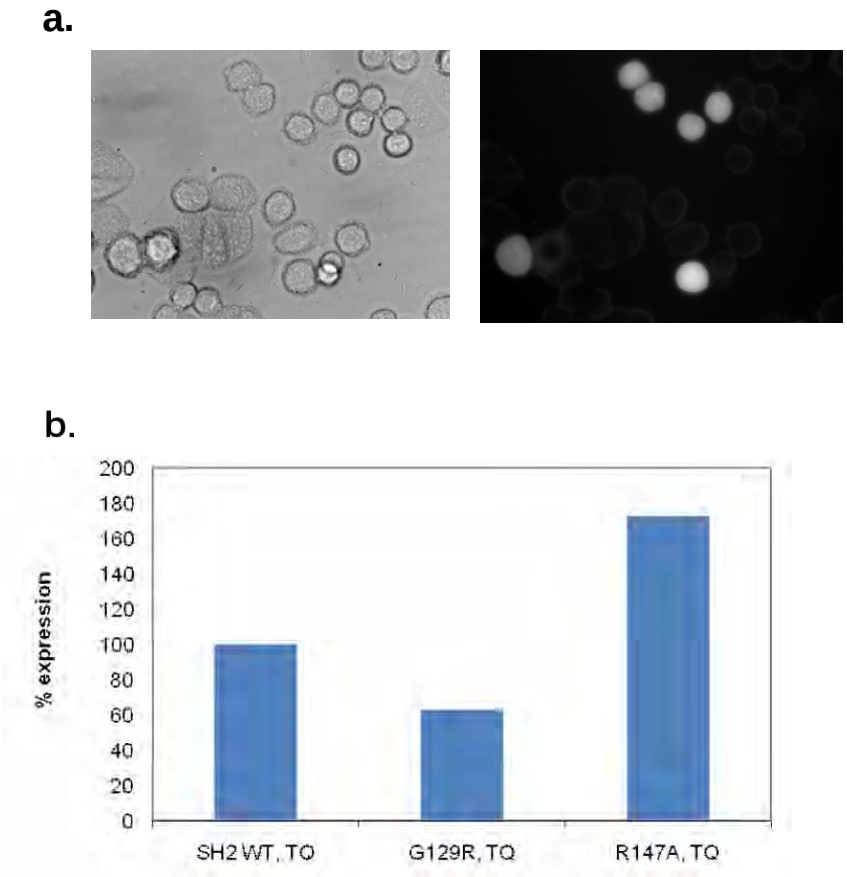

Figure 2. Expression of CHK constructs in breast cancer cells and expression profiles of CXCR4 in CHK mutants-expressing breast cancer cell lines

a. SKBR-3 breast cancer cells were transduced with MSCV-IRES-*GFP*-based retroviruses expressing wild-type SH2 CHK. Representative phase contrast (left panel) and *GFP*-expressed (*right panel*) images were photographed, b. Expression profiles of CXCR4 in CHK mutants-expressing breast cancer cell lines. Total RNA was extracted from the indicated cell lines, and quantitative real-time RT-PCR was performed as described in Figure 1 legend

b. Investigate whether G129R and R147A exert their effects through the transcription factor YY1

We have optimized gel shift assay conditions. During the extension period, we will investigate whether YY1 binding to the CXCR4 promoter is associated with the CHK-mediated CXCR4 regulation. For this purpose, we will perform gel mobility shift assay.

c. Perform in vivo metastasis assay using G129R- and R147A-infected breast cancer cells and assess metastasis

Our ultimate aim for this proposal is to investigate whether mutant CHKs modulate CXCR-4 mediated metastasis *in vivo*. We found CHK mutants modulate the expression of CXCR4 (Figure 2b). During the extension period, we will transplant CHK mutants-transduced breast cancer cells into NOD/SCID IL-2γ null mice and recipients will be screened by IVIS 200 imaging system at 6-8 weeks post transplantation.

Key Research Accomplishments

1. Our preliminary results indicate that CHK expression regulates the level of CXCR4 in breast cancer cell. Thus signaling pathways downstream of CHK and their cross talk are anticipated to control metastatic migration of breast cancer cells.

2. Our ongoing efforts have generated retroviral vectors carrying wild type (wt) and mutated (mt) CHK (wild-type SH2 domain, SH2-R147A and SH2-G129A) in which e GFP protein is bicistronically expressed as a marker. These materials will be used for the ongoing xenotransplantation experiments.

3. Although it has not been investigated in detail yet, the lack of a correlation between CXCR4 mRNA and protein in some breast cancer cell lines suggest that CXCR4 are regulated at the post-transcriptional level depend on cell type or cellular context.

Reportable Outcomes

There are no reportable outcomes yet because the experiments are still ongoing. We anticipate to publish one or two manuscripts depend on the results obtained during the NCE period.

Conclusion

CHK showed its ability to regulate CXCR4 mRNA, supporting our hypothesis that CHK signaling axis regulates the metastatic migration of breast cancer cells. Especially, wild-type SH2 domain, SH2-R147A and SH2-G129A displayed their differential capacity to regulate CXCR4 expression in breast

cancer cells. Their small size may allow for improved penetration into breast tumor tissue, although the further xenotransplantation experiments using the mutated CHK expressing breast cancer cell lines are necessary to verify the validity.

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Appendices

N/A